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A comparative study of several media for determining the bacterial content of milk by the plate count method

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A COMPARATIVE STUDY OF SEVERAL MEDIA FOR DETERMINING THE BACTERIAL CONTENT OF MILK BY THE PLATE COUNT METHOD

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INTRODUCTION

The nutrient agar plate count has been used for many years as a routine method of sanitary control of milk. It has been attacked by many workers on the grounds that the incubation period is too prolonged, that the method does not reveal the presence of pathogens, and that it does not allow the development of all types of organisms which are normally present in milk. The first objection can be met by pointing out that the sanitary quality of milk should not be judged by an analysis made at any one time but rather by a series of analyses over an extended time period. An individual count, therefore, means little. The second objection is met by recognizing the general fact that a clean milk will give a low count and a dirty milk a high count. It has been shown that a high-count milk is sometimes associated with illness (26). In other words, a high count may mean potential danger to health. Thus, a high count is an index, just as the colon organism is an index in water analysis.

The objection that cannot be answered easily is that the present method does not reveal all the organisms present in milk. This is undoubtedly true and has been recognized by the Standard Methods of Milk Analysis Committee, who answer this objection in an arbitrary manner.

The Standard Methods of Milk Analysis of the American Public Health Association (1928 Edition) (36) states: .... "it is not necessary in public health work that the counts used should represent the actual number of bacteria present or even that they should represent the greatest number of colonies that could be developed on agar media, if there is

a fairly accurate knowledge of the percentage developing ..... It has been abundantly demonstrated that no plate count, with any technic yet devised, gives the total number of bacteria in milk. Hence, the standard plate method should not be expected to show all the bacteria possible, but rather to furnish an artificial means by which different laboratories can get reasonably accurate, comparative results. Because some method gives a higher count than the standard technic does not prove that it is better for control work. The best count for the purpose would be the one giving the most uniform percentage of the actual number of bacteria."

Since we usually do not know the percentage of bacteria appearing on the nutrient agar plate, it is better to have the largest number developing that is possible. How can we have accurate and comparative results if some bacteria, important in sanitation, fail to develop? If a standard medium does not support the growth of some types of bacteria found in milk, a study of the nutritive deficiencies of the medium would remedy this situation. A medium could then be employed, that would, so far as possible, support the maximum number of bacterial colonies. If plate counts are to be carried on as a control on the cleanliness and efficiency of milk production, the maximum number of bacteria in milk should be enumerated.

Of course the ideal medium would seem to be milk agar, but there are so many difficulties associated with the preparation and use of a milk agar, that it would be unfit for use as a routine medium (20). Devereux (16, 17) has recently claimed that his yeast extract agar is a more efficient medium than the standard American Public Health Association medium for milk analysis.

Object of Investigation

The present study was undertaken with the purpose of determining whether Devereux's yeast extract formula was an efficient medium for milk analysis; first, by comparing the effect of the different constituents of the medium on the growth of certain of the bacteria commonly found in milk; and second, by comparing Devereux's medium with the standard medium, and with one based on the results of the first part of the experimental work. The plating of milk samples was the basis for this second comparison.

Review of Literature

The American Public Health Association appointed a committee in 1905 to formulate standard laboratory methods for the bacterial examination of milk. This committee found that the methods of milk examination prevalent varied due to four factors: 1. medium, 2. temperature of incubation, 3. period of incubation, 4. degree of shaking.

The first standard plating agar was originated by Marshall (21) and his associates and was designed to determine the initial contamination of milk from animal sources, especially from materials containing Escherichia coli. The originators of the method thought that the organisms of the Streptococcus lactis group were of no importance. The lactic acid organisms, however, are most important when the keeping quality of milk is considered. Heinemann and Glenn (22) concluded from their work that incubation at twenty degrees Centigrade was superior to incubation at thirty-seven degrees Centigrade because a higher and more accurate differential count was obtained. They preferred dextrose to lactose, and declared "the bacteriological examination of milk ought to be carried on with the object of improving the whole milk supply rather than a single source; therefore, the most accurate and scientific method of examination is the preferable one." Northrup and Farrand (24) tried to find the medium most favorable to the growth of bacteria in milk. They prepared media of agar, peptone, and salt in four portions and adjusted them to 5, 10, 15, and 20 degrees acid (Fuller's scale). The plates were incubated at twenty-one and thirty-seven degrees Centigrade. A bacterial count was taken at intervals to ascertain

the degree of acidity, percentage of lactose, and percentage of peptone most conducive to the growth of the milk bacteria. From sixty three per cent of the samples the best growth was obtained at twenty one degrees Centigrade; from nine per cent, at thirty seven degrees Centigrade; and from the rest, at either temperature. To check the results obtained by plating the miscellaneous milk organisms, pure cultures of bacteria commonly found in milk were grown on whey, standard, and four per cent lactose agar. The lactic acid bacteria grew equally well on standard and on lactose agar, while the "associative" bacteria grew best on the standard agar at thirty seven degrees Centigrade. The results were not especially marked in either case.

Sherman (32) prepared nutrient agars with reactions of plus 0.5, 1.0, and 1.5 (Fuller's scale). Lactose agar plates were incubated at thirty seven degrees Centigrade for forty-eight hours and average counts of ten plates each from fifteen samples of milk showed much lower counts with the 1.5 medium than with the others. Sherman recommended a plus 0.5 medium for milk analysis. Faber (18) attached no significance to reactions between pH 6.2 and pH 7.0, although he made the statement that pH 6.8 was best for raw milk, and pH 6.4 for pasteurized milk.

Sherman (33) compared counts on standard agar and on one per cent lactose agar using triplicate plated incubated at thirty seven degrees Centigrade for forty-eight hours. Results from eighteen samples of raw market milk showed an increase of forty three percent in counts when lactose was added to the medium. An increase in the size of colonies was also noted.

Robertson (29) found that as the period of incubation was lengthened at a temperature of twenty-one degrees Centigrade, the ratio that occured most frequently between the direct microscopic count and the plate count approached, though it did not reach, unity. The use of lactose agar with an incubation period of five days at twenty-one degrees Centigrade reduced the number of widely discrepant counts to the lowest number observed in any of these comparisons. The results of the studies justified, according to Robertson, the criticism that has always existed against the accuracy of the agar plate counts, and especially counts from plates incubated for two days at thirty-seven degrees Centigrade.

Slack (35) found that beef infusion in media was very efficaceous, and deplored the change to beef extract. The reports of various workers upon the bacterial count of milk samples agreed within a range of six per cent.

Sears and Case (31) agreed that the present standard medium was not a favorable medium for the growth of certain types of bacteria found in milk. The small quantity of milk added to the medium in plating low dilutions supplied the nutritive deficiency. More consistent results were obtained, they declared, if dilutions of samples higher than 1:100 were made in sterile 1:100 milk.

In 1920 Ayers and Mudge (1) gave the formulae for three agar media. Two of these media contained skimmed milk powder with different amounts of peptone and meat extract. The third contained skimmed milk powder and yeast extract, no peptone or meat extract being used. They claimed that this avoided variations in the composition of peptone and meat extract. The counts on milk agar were higher and the colonies larger

than on the standard extract medium. Counts were also higher from milk powder agar than from standard infusion agar. On milk powder-yeast agar the counts may be lower. Ayers and Mudge claimed that they were able to make qualitative distinctions of the colonies on the milk agar plate.

Bolling (4) declared that the Ayers' medium is much better than the standard medium. No experimental results were reported, however, as a basis for these statements. One of the men on his committee on Methods of Bacterial Analysis of Milk and Milk Products, Cooledge, who developed a method of checking the keeping quality of milk by its colorimetricly determined H-ion concentration, found that milk agar counts checked more closely with the actual keeping quality of the milk. Bolling believes that lactose is a necessary ingredient in standard medium for milk analysis. Borck (7) also found milk agar to be superior to meat extract agar. Parker and Beyers (27) compared the 1920 American Public Health Association standard agar with Ayers' milk-powder agar A. They declared that the colonies on milk agar at the end of forty-eight hours were larger and more easily counted than those on meat extract agar. With fresh raw milk giving counts of under 10000 colonies per c.c. there was no material difference in the two agars. With raw milk of over 50000 colonies per c.c. milk agar gave higher counts. On pasteurized milk, Ayers' milk agar gave higher counts with considerable regularity, and with improperly pasteurized milk the counts were enormously higher.

Supplee, Whiting, and Downs (38) reported that plain agar at thirty-seven degrees Centigrade for forty-eight hours were unquestionably the least favorable medium and temperature for milk plates; the use of lactose agar at this

temperature appeared to have few if any advantages over plain agar, and although dextrose agar at thirty-seven degrees Centigrade had distinct advantages over the other media, nevertheless, the majority of the results obtained from it were lower than those produced at twenty or thirty degrees Centigrade for five days. For developing the maximum counts, dextrose agar at thirty degrees Centigrade for five days seemed to be superior to any of the other combinations considered in the work of these investigators. This medium at twenty degrees Centigrade for five days was also preferable to plain agar or lactose agar at either twenty or thirty degrees Centigrade. The investigators stated that counts obtained at thirty-seven degrees Centigrade after forty-eight hours are probably subject to greater discrepancies than those obtained at the lower temperatures for longer periods of time.

* Pederson (28) determined the effect of the temperature of incubation upon the agar plate count of milk and pointed out that a maximum count and a reduction in the size of errors was obtained at lower incubation temperatures. He found this temperature to be approximately thirty-two degrees Centigrade.

Supplee and Flanigan (37) compared various media. Taking the averages of all results obtained from standard meat extract agar as 100 per cent, other media gave the following results: Standard infusion agar, 124.4 per cent, Bacto dehy-

* This work was published after our investigation was completed.

drated agar with milk, 153.2 per cent; dehydrated milk agar, 167.6 per cent; milk agar (Ayers' spray process), 170.9 per cent; milk agar (Just process), 177.2 per cent. These results agreed with Supplee, Whiting, and Downs [illegible] lactose agar, and indicated the superiority of milk agar over other media used.

Bolling (5) declared: "If bacterial counts of milk are to record, as nearly correctly as is possible with a single medium, the bacterial content of the milk under examination, lactose appears to be a vitally necessary ingredient. The organisms developing best upon lactose certainly exercise the utmost influence upon the keeping qualities of milk and their inclusion is essential in a plate count. The present standard meat extract agar frequently either does not show these colonies at all within the forty-eight hour incubation period or they are so minute as to be overlooked."

Zoller (43) found that in every instance milk powder agar gave a higher total count than the standard agar. He previously had shown that a higher grade pasteurized milk showed a predominance of acid forming colonies on the plate made from milk powder agar. The milk powder agar was easily prepared in modified form and yielded a higher total count than standard agar. The milk agar, he also declared, permitted qualitative differentiation on plates. This confirmed Ayers' work.

Frobisher (20) used standard medium, lactose agar, milk powder agar, milk powder-yeast agar, and milk powder agar with one per cent lactose. All of the enriched media gave a higher total count than standard medium. Colonies were larger, more uniform, and more regular. The lactose agar was as uniform in composition as the present standard agar. He reported that

all of the enriched media made possible a differentiation of bacterial species.

Norton and Seymour (25) reported that in regard to uniformity of results on five media, veal infusion was first, meat extract second, dehydrated agar third, peptonized milk fourth, and Ayers' milk agar fifth. They found the actual differences to be small and the counts were not comparable. They were not satisfied with Ayers' media, although from their results, it appeared to be very satisfactory with pasteurized milk. The authors stated: "Discordant results in counting bacteria in milk by the agar plate method are due to errors in technic and not inherent in methods or media recommended." This last statement is quite at variance with the results of all the other workers cited in this paper.

Swenarton (39) found that pin-point colonies in milk supplies in Baltimore were due to streptococci, but gave no indication as to their growth requirement.

Fabricius and Hammer (19) showed that the addition of one per cent sucrose to standard medium brought about a closer check between different dilutions, increased the size of colonies, and reduced the number of pin-point colonies in plates from ice cream. This medium has been adopted at the Iowa Experiment Station for the routine plating of ice cream.

Devereux (16) described a medium containing yeast extract, peptonized milk, dextrose, and salt, which he claimed was excellent for the cultivation of organisms occurring in milk. He later (17) brought out figures tending to show a superiority of the yeast extract medium over the standard medium. He claimed that counts were obtained at the

end of twenty-four hours which were, on the average, comparable to counts on plain nutrient agar made at the end of forty-eight hours. At the end of forty-eight hours the yeast extract agar gave counts which were on the average forty-five per cent higher than similarly made counts on standard agar.

Bowers and Hucker (8) substituted yeast extract for beef extract in the standard medium and made comparisons between the two media. They also observed the effect of modifications of the standard medium on the number and types of bacteria in milk. These modifications were standard agar with and without dextrose, plain peptone agar, yeast agar with and without dextrose, beef and yeast extract agar with and without peptone, and Difco yeast dextrose agar which contained beef extract, yeast extract, tryptophane broth, peptone, and dextrose. Incubation was at thirty-two and thirty-seven degrees Centigrade. Bowers and Hucker concluded that yeast extract was not superior to beef extract for plating either raw or pasteurized milk; the combination of yeast and beef extract in a medium for milk plating was more efficient than the use of either one separately; neither yeast extract, nor beef extract materially, increased the number of colonies over plain peptone agar; the addition of a fermentable carbohydrate to the standard medium generally caused an appreciable increase in count and colony size; and the addition of glucose to the standard medium increased the count approximately two and a half times more than the addition of yeast extract. They stated "Counts obtained with the present standard temperature may give a false impression of the actual number of bacteria present in milk."

Much doubt has been cast on the usefulness of the agar plate technique, because of its shortcomings, as evidenced by the literature. Several investigations by workers in this field have helped to restore faith in the method.

Conn (14) found that if the standard procedures were used, consistent counts could be secured in different laboratories.

Hastings (21) declared, "The routine examination of milk should give the maximum amount of information concerning the number and kind of bacteria present." He stated that the bacterial content of any sample of milk depends on the initial contamination and subsequent bacterial growth. He stated further that differentiation of colonies is practically impossible in routine milk analysis.

Breed and Stocking (10) stated ------"skilled analysts using the proper technique ordinarily obtain reasonably accurate estimates of the number of bacteria in milk by the plate method, provided the milk contains isolated organisms of a type capable of growth on agar under the conditions maintained."

Supplee, Whiting, and Downs (38) decided that possible variations in bacterial counts resulting from the present plate method of enumeration should not be considered as a condition eliminating its usefulness. Furthermore they stated---------"bacterial counts together with the discrepancies to which they are subject, should be considered only with full knowledge of their limitations and of the fact that they constitute but one item of the evidence necessary to grade classes according to its wholesomeness and keeping

qualities. In order to harmonize these variations with existing numerical bacterial standards, it is essential that all factors tending to cause variations and discrepancies be reduced to a minimum."

Cooledge (15) declared that when plating methods are used, no one medium should be expected to give results which indicate exactly the condition of all grades of milk. He found that higher counts kept pace with more accurate plate methods. Variations ranged from 45.8 per cent in counts under 25000 per c.c., to 97.0 per cent in counts over one million per c.c. The medium giving the highest average counts depended on the bacterial groups predominating in the sample. This might account for the widely divergent results reported.

Breed (9) declared that there is a most important need for standardization of composition and reaction of media.

Wright and Thornton (41) made 2330 counts of milk plates poured in duplicate series, 100 plates to the series, in order to determine how accurate the plate count could be. They reached the conclusion that plate counts were no more accurate than other laboratory methods available for judging the quality of milk.

Brew (11), discussing the comparative accuracy of direct microscopic and agar plate methods in determining numbers of bacteria in milk, said that the addition of lactose reduced variability in counts and increased the correlation between the direct microscopic and agar plate counts.

Baker (2) commenting on the data presented by Brew and Dotterrer (12) on bacteria counts from milk, declared that if the direct microscopic method really gives an esti-

mate of the number of bacteria, then the plate method underestimates the number of bacteria in almost all milk giving a direct microscopic count of over 180000 bacteria per c.c. Both methods are subject to errors and it is very desirable to know these errors in order to judge the significance of differances in counts. Robertson (30) gave six major uncontrollable factors: (1) uneven distribution of bacteria in milk, (2) medium does not support growth, (3) clumping, (4) unfavorable incubation time, (5) unfavorable temperature of incubation, (6) personal error. Bolling (6) declared that, "it has become apparent to many of us that one of the most prolific sources of error lies in the character of the medium employed both in official and dairy plant laboratories."

The literature may be briefly summarized by stating that for the most part there is marked dissatisfaction with the present method of milk analysis by the agar plate method. The consensus of opinion is that an incubation temperature lower than thirty-seven degrees Centigrade is desirable, and that the present standard medium gives false results in terms of the numbers and types of organisms in milk. The addition of a fermentable carbohydrate causes an increase both in count and in colony size. There is a general feeling that the agar plate method is very useful, and that any limitations in its use should be overcome. The results of the earlier investigators are not comparable with present results, because, first, the reaction of the medium was expressed in terms of the Fuller scale which cannot be correlated in terms of pH; and second, the statistical analyses of their results are of questionable value because of what Robertson has recently

poimted out, that bacterial counts should not be averaged arithmetically as older workers did. In consideration of this view point their results are to be accepted with considerable doubt.

EXPERIMENTAL WORK

PART I
Studies on the effect of the constituents of Deveraux's medium on growth

It has long been known that the standard medium does not support the growth of all types of organisms found in milk. As a rule the pathogenic bacteria are not especially sought for in the routine plate analysis. The problem then resolves itself into finding the peculiar conditions of growth of the common milk-borne organisms. These conditions are nutrients, pH, time and temperature of incubation, surface tension, oxidation-reduction potential, and buffering capacity of the medium, all of which factors might influence bacterial growth.

Since Deveraux has claimed his medium to be very satisfactory for the growth of milk bacteria, it was decided to incorporate the ingredients of the medium into standard agar in such a manner as to bring out the factor responsible for any influence on bacterial growth. These media were then used in plating some of the common milk organisms. Since lactose had been used by many investigators, and because of its presence in milk, it was decided to incorporate it also in the studies. The criterion of efficiency was to be the amount of growth as measured by colony size, and production of acid in order to determine whether there was inhibition of growth by production of acid by the organisms.

A temperature of thirty degrees Centigrade was used for incubation because the literature and previous experience indicated that this was a more suitable temperature for milk control work. xxxx The majority of the organisms dealt with are from soil, air, and water, which have their

optimum temperature for growth at, or near, thirty degrees Centigrade, rather than at thirty seven degrees Centigrade. It must not be forgotten that milk is examined for its sanitary quality rather than for its pathogenic bacteria content.

A. Methods and procedure.

The following media were employed in the effort to show the effect of each constituent of the Devereux medium on bacterial growth:

A. Standard agar.
B. Standard agar plus one per cent dextrose.
C. Standard agar plus 0.5 per cent yeast extract.
D. Standard agar plus 0.5 per cent yeast extract and one per cent dextrose.
E. Devereux's medium.
F. Standard agar plus one per cent lactose.
G. Standard agar plus one per cent lactose and 0.5 per cent yeast extract.
H. Devereux's medium with the substitution of lactose for destrose.

The composition of each of these media is shown in table I.

The standard medium was made according to the directions contained in the standard Methods of Milk Analysis, Fifth Edition,(36). Devereux's (16) medium contains 5 grams of yeast extract, 10 grams of peptonized milk, 5 grams of salt, 15 grams of agar, and one liter of distilled water. Five cc. of a 0.05 per cent alcoholic solution of brom thymol blue per liter was added to each medium to indicate changes in reaction during incubation.

TABLE I.

Media Used in the Studies.

Medium	Code Letter	pH	Ingredients per liter in grams							
			Beef Extract	Peptone	Dextrose	Lactose	Yeast Extract	Peptonized Milk	Salt	Buffered Salts
Standard nutrient agar	A	6.6-6.8	3	5						
Standard plus dextrose	B	6.8	3	5	10					
Standard plus yeast extract	C	6.8	3	5			5			
Standard plus yeast extract and dextrose	D	6.8	3	5	10		5			
Devereux's medium	E	7.0			10		5	10	5	
Standard plus lactose	F	6.8	3	5		10				
Standard plus lactose and yeast extract	G	6.8	3	5		10	5			
Modified Devereux	H	7.0				10	5	10	5	
Standard plus buffer	B_1	6.8	3	5						.1 K_2HPO_4
Devereux plus buffer	E_1	7.0			10		5	10	5	.1 K_2HPO_4
Modified Devereux plus buffer	H_1	7.0				10	5	10	5	.1 K_2HPO_4
New medium	X	6.8-7.0	3	5	1	5				.1 KH_2PO_4 and .2 $Na_2HPO_4 12H_2O$

The procedure used in determining the effect of these media on growth was to take a loopful of the organism u under test from a forty-eight hour nutrient agar slant, shake in a 99 c.c. saline blank for five minutes, and then make appropriate dilutions. The dilution to be used was estimated at first by the Wright (41) method , using the haemocytometer, but it was found after a few trials that a correct dilution could be estimated from the turbidity of the saline mixture. Using the same loop throughout the project, and the same amount of inoculum as nearly as could be judged, the usual dilution employed was 1 to 10^6. This gave about twenty colonies on the plate when one c.c. was used as an inoculum, thus preventing over-crowding, so that the effect of the medium only would be observed. Duplicate plates were poured, one set being incubated at thirty degrees and the other at thirty-seven degrees Centigrade for forty-eight hours.

The organisms were taken from the stock cultures of the Department of Bacteriology and Physiology of the Massachusetts State College, with the exception of several unnamed streptococci of the Streptococcus lactis type isolated from sour milk. The organisms can be divided generally into three groups, the subtilis, coli-aerogenes, and a group of miscellaneous cocci commonly found in milk.

These data are presented in Tables II, III, and IV.

Effect of Experimental Media upon the Subtilis Group of Organisms.

Table II	B. subtilis		B. mycoides		B. megatherium		B. mesentericus		B. tumescens		B. simplex		B. cereus		B. fusiformis	
	30° G	37° R	30° G	37° R	30° G	37° R	30° G	37° R	30° G	37° R	30° G	37° R	30° G	37° R	30° G	37° R
Standard A	4	-	4	-	4	-	4	-	3	-	3	-	4	-	3	-
Standard plus dextrose B	1	+	1	+	1	+	1	+	2	+	1	+	1	+	1	+
Standard plus yeast extract C	4	-	4	-	4	-	4	-	3	-	3	-	4	-	2	-
Standard plus yeast extract and dextrose D	4	-	4	-	1	+	4	-	3	-	1	-	4	-	2	-
Standard plus lactose F	4	-	4	-	4	-	4	-	3	-	3	-	1	+	3	-
Standard plus lactose and yeast extract G	4	-	4	-	4	-	4	-	3	-	3	-	4	-	3	-
Devereux's medium E	1	+	1	+	1	+	1	+	1	+	1	+	1	+	1	+
Modified Devereux H	1	+	1	+	1	+	1	+	1	+	1	+	1	+	1	+

+ = acid; - = alkaline to neutral; 4 = very good growth; 3 = good growth; 2 = fair growth; 1 = poor growth.

Analysis of Table II

The organisms in the subtilis group need not be discussed individually, since they differed so little from each other in their behavior. They all grew well on the standard medium; on dextrose agar the colonies were all quite small and there was a very distinct acid reaction imparted to the medium. The growth on standard agar plus yeast extract did not differ from that on standard agar. The standard agar plus yeast extract plus dextrose produced variable results. Bacillus subtilis, Bacillus mycoides, Bacillus cereus, and Bacillus tumescens grew as well on this medium as on the standard agar. Bacillus megatherium and Bacillus simplex colonies were reduced in size on the standard agar plus yeast extract plus dextrose. On Devereux's agar colony growth was uniformly very small. All of the organisms employed grew well on the lactose medium, growth being equal to that on the standard agar. The growth on standard agar plus lactose plus yeast extract was also excellent. The growth on modified Devereux's agar was as poor as on the Devereux medium.

There is a curious fact which on the face of it seems inexplicable, namely, that on the dextrose agar (medium B) the size of the colonies was inhibited. It was thought at first that this might be due to surface tension phenomena, but examination of the different media by the stalagmometer showed that there was no correlation between the surface tension of the medium and inhibition of the colony size. There was, however, a marked correlation between production of acid, in the medium, as measured by the bromthymolblue indicator, and the size of the colonies. If

the production of acid had an inhibiting effect on growth, the use of a carbohydrate medium, especially if poorly buffered, would tend to produce variable effects, depending on the acid tolerance of the organism employed in this connection. A study was made of the acid tolerance of the organisms under test. This will be discussed later, (p.29).

Yeast extract alone had no effect on these organisms, but in medium D it seemed to act as a buffer since there was no inhibition of growth,or acid production even though dextrose was present. This indicates that the standard medium must be buffered to a greater extent than at present if a fermentable sugar is to be added. A probable explanation for the behavior of the organisms in Devereux's medium is that the production of acid from dextrose coupled with a poor buffering system caused inhibition. In media F and G the lactose was used in such small quantities that the buffer present was able to neutralize the small amount of acid formed.

An explanation of the phenomenon of acid inhibition is indicated by Shrader (34) who investigated the availability of the amino acids in peptone. He was able to correlate the facts that Berman and Rettger (3) had pointed out. These investigators showed that the H-ion concentration plays an important in inhibiting nitrogen metabolism in a medium which contains a fermentable sugar. The failure of bacteria to attack a protein was dependent on a coincident rise in acidity of the medium, but when K_2HPO_4 was added as a buffer, protein metabolism became as marked as in plain peptone broth. Thus the failure of certain organisms to

attack complex nitrogenous bodies in a media containing carbohydrate is due to the accumulation of products inhibitory to growth but which may be neutralized by buffers. Carbohydrates supply the energy, and nitrogen the growth.

To determine if the addition of a buffer would remedy any cases of inhibition due to acid production, three new media were made up. They were simply media B, E, and H with the addition to each of 0.1 gram of K_2HPO_4 per liter. They will be referred to as media B_1, E_1, and H_1. These media were added to the others employed in the experiments immediately following.

TABLE III

Effect of Experimental Media upon the Coli-Aerogenes Group.

		Aerobacter aerogenes				Escherichia coli			
		30 degrees C.		37 degrees C.		30 degrees C.		37 degrees C.	
		growth	reaction	growth	reaction	growth	reaction	growth	reaction
Standard agar	A	2	-	3	-	2	-	3	-
Standard plus dextrose	B	1	+	2	+	1	+	2	+
Standard plus yeast extract	C	2	-	3	-	2	-	3	-
Standard plus dextrose and yeast extract	D	2	-	3	-	2	-	3	-
Standard plus lactose	F	4	-	4	-	4	-	4	-
Standard plus lactose and yeast extract	G	3	-	3	-	3	-	3	-
Devereux's medium	E	3	-	3	-	3	-	3	-
Modified Devereux's medium	H	3	+	3	+	3	+	3	+
Standard plus dextrose and buffer	B_1	1	+	2	+	1	+	2	+
Devereux's plus buffer	E_1	2	-	2	-	2	-	3	-
Modified Devereux's plus buffer	H_1	3	-	3	-	3	-	3	-

4= very good growth
3= good growth
2= fair growth
1= poor growth

\- = alkaline to neutral

\+ = acid

Analysis of Table III

The coli-aerogenes group grew well on the standard medium, but poorly on the standard medium plus dextrose. The addition of yeast extract to the standard medium caused no additional growth beyond that on the standard medium. The same was true when yeast extract and dextrose were added to the standard medium. This group grew much better on Devereux's medium than on any of the media previously discussed. The best growth was obtained on standard agar plus lactose. The standard agar plus lactose plus yeast extract was equal to Devereux's medium in growth promoting qualities. The addition of the buffer did not result in any change in growth on the standard agar plus dextrose, Devereux's medium, or modified Devereux's medium.

These experiments show that dextrose and yeast extract do not stimulate the growth of this group, and that lactose is the more desirable ingredient to add to the standard medium in order to promote growth and to increase colony size. In this group we also have the same phenomenon that Berman and Rettger observed, namely, that a rise in H-ion concentration leads to an inhibition of nitrogen metabolism and a consequent inhibition of growth. Thirty-seven degrees Centigrade was a more favorable temperature than thirty degrees Centigrade for incubation.

Effect of Experimental Media upon Miscellaneous Cocci found in Milk.

TABLE IV

	Sarcina lutea		Staph. aureus		Strep. lactis		Micr. varians		Staph. albus		Micr. tetragena		Micr. cereus		Strep. pyogenes		Staph. citreus		Pseudo. fluorescens	
	30 G	37 R	30 G	37 R	30 G	37 R	30 G	37 R	30 G	37 R	30 G	37 R	30 G	37 R	30 G	37 R	30 G	37 R	30 G	37 R
Standard agar	2 -	1 -	1 -	1 -	1 -	1 -	1 -	1 -	1 -	1 -	1 -	1 -	2 -	1 -	1 -	1 -	1 -	1 -	1 -	1 -
Standard plus dextrose	1 +	1 +	1 +	1 +	1 +	1 +	1 +	1 +	1 +	1 +	2 +	2 +	1 +	1 +	2 +	2 +	1 +	1 +	1 +	1 +
Standard plus yeast extract	3 -	2 -	1 -	1 -	1 -	1 -	1 -	1 -	2 -	2 -	1 -	1 -	1 -	1 -	2 -	2 -	1 -	1 -	1 -	1 -
Standard plus dextrose and yeast extract	1 +	1 +	1 +	1 +	1 +	1 +	1 +	1 +	1 +	1 +	2 +	2 +	1 +	1 +	2 +	2 +	1 +	1 +	1 +	1 +
Standard plus lactose	3 -	3 -	2 -	2 -	2 +	2 +	2 -	2 -	2 +	2 +	3 +	3 -	3 ±	3 ±	3 ±	3 ±	2 -	2 -	1 -	1 -
Standard plus lactose and yeast extract	3 -	2 -	2 -	2 -	1 +	1 +	1 -	1 -	3 +	3 +	2 +	2 +	2 +	2 +	2 +	2 +	1 -	1 -	1 -	1 -
Devereux's medium	3 -	2 -	1 +	1 +	1 +	1 +	2 +	2 +	1 +	1 +	2 +	2 +	2 +	2 +	2 +	2 +	1 +	1 +	1 +	1 +
Modified Devereux's medium	1 +	1 +	1 -	1 -	1 +	1 +	2 -	2 -	2 +	2 +	2 -	2 -	1 -	1 -	2 +	2 +	1 ±	1 ±	1 -	1 -
Standard plus dextrose and buffer	1 +	1 +	1 -	1 -	1 -	1 -	2 -	2 -	2 -	2 -	2 -	2 -	2 -	2 -	2 -	2 -	1 -	1 -	1 -	1 -
Devereux plus buffer	2 -	2 -	1 +	1 +	1 +	1 +	2 +	2 +	1 +	1 +	2 +	2 +	1 +	1 +	2 +	2 +	2 -	2 -	1 +	1 +
Modified Devereux plus buffer	2 -	2 -	1 -	1 -	1 +	1 +	2 -	2 -	3 +	3 +	2 -	2 -	1 -	1 -	2 +	2 +	2 -	2 -	1 -	1 -

+ = acid; - = alkaline to neutral; ± = faint acid; 4 = very good growth; 3 = good growth; 2 = fair growth; 1 = poor

Analysis of Table IV

Table IV shows the growth of the miscellaneous cocci found in milk.

1. Sarcina lutea grew fairly well on the standard medium. The addition of dextrose to the standard medium decreased the amount of growth. The addition of yeast extract to the standard medium caused a slight increase in growth over that on the standard medium. The same growth was observed on the standard agar plus dextrose plus yeast extract as on the standard agar plus dextrose. Devereux's medium showed the same growth-promoting qualities as the standard agar plus yeast extract. The growth on the standard agar plus lactose, and the standard agar plus lactose plus yeast extract, was equivalent to that on Devereux's medium. Growth on the modified Devereux's medium was as poor as on the standard agar plus dextrose medium. The addition of a buffer aided growth on the modified Devereux's medium slightly, but not on the standard agar plus dextrose nor on the Devereux medium. An incubation temperature of thirty degrees Centigrade was better than thirty seven degrees Centigrade.

From this information it is obvious that: (1) dextrose alone is not of much value for promoting growth of this organism; (2) yeast extract is of some value; (3) yeast extract plus dextrose is of no value; (4) Devereux's medium is equivalent to the standard agar plus yeast extract; (5) lactose, and lactose plus yeast extract are equivalent to Devereux's medium; (6) modified Devereux's medium is as poor as the standard agar plus dextrose; or the standard agar plus dextrose plus yeast extract; (7) the addition of

a buffer did not stimulate growth to any great extent. The important fact in this case is that lactose gives an appreciable increase in growth, equivalent to that on the Devereux medium.

2. Staphylococcus aureus grew poorly on all media except the standard agar plus lactose,and the standard agar plus lactose plus yeast extract. Yeast extract was of no value for promoting growth as the organisms grew poorly on the standard agar plus yeast extract. The addition of a buffer aided only in changing the reaction from acid to alkaline on the standard agar plus dextrose. Growth was not increased, however. Thirty degrees Centigrade was the better temperature of incubation. It was evident that lactose was the only material used that had any influence in increasing growth.

3. Streptococcus lactis grew very poorly on all the media except the standard agar plus lactose. This organism is very important in milk sanitation as it gives a very reliable index of the age of the milk and the conditions of holding. Neither the Devereux nor the standard media were of no value in stimulating growth of this organism. The addition of lactose aided greatly. Thirty degrees Centigrade was a much better temperature of incubation than thirty-seven degrees Centigrade.

4. Micrococcus varians grew poorly on standard agar, standard agar plus dextrose, and standard agar plus dextrose plus yeast extract, but grew much better on Devereux's medium and modified Devereux's medium. The growth was poor on standard agar plus lactose. The addition of a buffer aided growth on standard agar plus dextrose, Devereux's agar, and modified Devereux's agar. These facts

indicate that peptonized milk is the factor that stimulates growth. This is the first and the only organism that is definitely stimulated by peptonized milk. A buffer also aids growth.

5. Staphylococcus albus grew poorly on standard agar and standard agar plus dextrose. The addition of yeast extract to the standard medium stimulated growth, but the addition of dextrose to standard agar plus yeast extract decreased growth to the level of the standard medium. Devereux's medium was as poor as the standard medium in growth promoting qualities. Growth on the lactose medium was as good as on the standard agar plus yeast extract, while the addition of yeast extract to the lactose medium produced very good growth. On the modified Devereux growth was equal to that on standard agar plus lactose. The addition of a buffer aided growth on standard agar plus dextrose and greatly aided growth on the modified Devereux medium. It seems evident that this organism is stimulated by lactose and by yeast extract either alone or in combination.

6. Micrococcus tetragenus grew poorly on standard agar. The addition of dextrose caused an increase in colony size. Yeast extract produced no growth increase over that on the standard medium. This organism grew as well on standard agar plus dextrose plus yeast extract as on standard agar plus dextrose. The growth on Devereux's medium was equal to that on standard agar plus dextrose. The addition of lactose to standard agar provided the largest colonies observed on any of the media. The addition of yeast extract to the lactose medium resulted in a smaller colony growth than on the standard agar plus lactose. Growth on

the modified Devereux medium was the same as on the standard agar plus lactose plus yeast extract. The addition of a buffer to standard agar plus dextrose caused a change in reaction from acid to alkaline, and an increase in the size of the colonies. Buffering the Devereux and the modified Devereux media had no effect on the size of the colonies.

These facts indicate that for this organism the addition of dextrose increased growth, but not to the extent that lactose did. Yeast extract had no effect on growth. Thus, the beneficial effect of Devereux's medium seems to be due largely to the presence of dextrose. Buffering the media which contained fermentable carbohydrates aided growth by keeping the H-ion concentration down. This organism grew better at thirty degrees Centigrade than at thirty-seven degrees Centigrade.

7. Micrococcus cereus grew fairly well on the standard medium, but there was less growth on standard agar plus dextrose, standard agar plus yeast extract, and standard agar plus dextrose plus yeast extract. On the lactose medium the growth was equivalent to that on standard agar. The best growth was obtained on standard agar plus lactose. The growth on standard agar plus lactose plus yeast extract was somewhat less than the growth on standard agar plus lactose. The same growth was observed on the modified Devereux medium as on the standard agar plus dextrose. The addition of a buffer caused an increase in growth on standard agar plus dextrose, but not on any of the other media. The addition of dextrose inhibited growth while the addition of lactose increased growth. Yeast extract had no effect. Devereux's medium was no better than standard

medium. Buffering increased the growth-promoting efficiency of the dextrose medium only.

8. Streptococcus pyogenes grew poorly on standard agar; it grew better on all other media. The addition of a buffer to the standard agar plus dextrose produced a change in reaction from acid to alkaline. All other carbohydrate-containing media became acid in reaction. It can therefore be inferred that standard agar does not contain the right type of nutriment necessary for the growth promotion of this organism. Lactose or dextrose, alone or in combination with yeast extract or beef extract and a buffer, stimulated growth. Thirty degrees Centigrade was as good an incubation temperature as thirty-seven degrees Centigrade.

9. Staphylococcus citreus grew poorly on standard agar, standard agar plus dextrose, standard agar plus yeast extract, standard agar plus yeast extract plus dextrose, and Devereux's medium ; it grew much better on standard agar plus lactose. It did not grow as well on standard agar plus lactose plus yeast extract, or on modified Devereux's medium. The addition of a buffer to the standard agar plus dextrose did not aid growth, but it did change the final reaction from acid to alkaline. The addition of a buffer to Devereux's and to modified Devereux's media aided in promoting growth. Of all ingredients used lactose is the one that most definitely stimulated growth. Buffering changed the final reaction of standard agar plus dextrose from acid to alkaline but did not increase bacterial growth. Buffering did increase growth on the Devereux and modified Devereux media.

10. Pseudomonas fluorescens, which is included here, although it is not a coccus, grew poorly on all media. It did not produce acid on the lactose agar. Since it has been shown that the production of acid exerts a deletorious effect on some of the organisms used in this investigation, the lactose agar would be more satisfactory than any of the other carbohydrate media, because the Pseudomonas fluorescens, if present in any considerable numbers in milk, would not produce acid on lactose agar, and consequently would not be a means of inhibiting some other organism not aciduric.

A general summary of the experiments conducted on this group indicates that dextrose is valuable in promoting growth of bacteria in a few cases, and especially when buffered. Lactose promoted growth in all cases. Yeast extract promoted growth in a few cases but exerted no effect in the majority of cases. The same can be said of the Devereux medium. Thirty degrees Centigrade was equal to, or better than, thirty-seven degrees Centigrade as a temperature of incubation.

In order to determine positively if there was any correlation between the production of acid and inhibition of growth, it was necessary to determine the approximate pH at which there was inhibition. The acid tolerance of the organisms was tested by streaking them from a saline suspension, on a standard nutrient agar plate which had been adjusted to the desired pH.

These data are presented in Table V.

TABLE V

EFFECT OF pH ON GROWTH

Organism	pH of nutrient agar 7.0	6.0	5.0	4.0
Bacillus mycoides	2	2	-	-
Bacillus subtilis	1	1	-	-
Bacillus cereus	2	2	-	-
Bacillus simplex	2	2	-	-
Bacillus megatherium	2	2	1	-
Bacillus mesentericus	2	2	-	-
Bacillus tumescens	2	2	-	-
Bacillus fusiformis	2	2	1	-
Bacillus ruminatus	2	1	-	-
Escherichia coli	2	2	1	-
Pseudomonas fluorescens	2	2	1	-
Staphylococcus albus	2	2	1	-
Staphylococcus citreus	2	1	1	-
Micrococcus flavus	2	-	-	-
Sarcina lutea	2	2	2	-
Aerobacter aerogenes	2	2	2	-
Micrococcus cereus	2	2	2	-
Gaffkya tetragena	2	2	2	-
Staphylococcus aureus	2	2	2	-

2 good growth

1 poor growth

- no growth

ANALYSIS OF TABLE V

The results of this table can be correlated with acid inhibition only in a general way because the pH of inhibition was not determined exactly and the H-ion concentration produced by the organisms was stated only as acid or alkaline. It can be stated however, after an inspection of the table, that those organisms which were inhibited between pH 6.0 and pH 5.0 were inhibited by the production of acid. For the most part, these were organisms of the subtilis group. The organisms which were inhibited between pH 5.0 and pH 4.0 were not usually inhibited by production of acid. It seems that an aciduric organism has the ability to attack nitrogenous bodies, even with the rise in H-ion concentration. The non-aciduric organisms do not have this power, and consequently some means must be provided for preventing a rise in H-ion concentration during incubation.

PART II
Studies on plating milk samples with new media.

After the work of Part I was completed, the next step was to devise a new medium, based on the experience of the previous work, and then to measure its efficiency by plating milk samples on it and comparing with the standard medium and the Deveraux medium. Both raw and pasteurized milk were used and the plates incubated at temperatures of thirty and thirty-seven degrees Centigrade.

Since yeast extract and peptonized milk had failed to show any decided effects on bacterial growth it was decided to keep peptone and beef extract as the sources of nitrogen and salts. The peptone has a higher percentage of nitrogen than peptonized milk and yeast extract, there being 16.3 per cent in the former and 13.7 per cent in the latter. Lactose was added but in 0.5 per cent concentration instead of 1.0 per cent. One tenth grams of KH_2PO_4 and 0.2 grams of Na_2HPO_4 were used as additional buffers. They were added in the amounts prescribed in order that there might be no disturbance of the desired reaction of the medium, i.e. pH 6.8 to pH 7.0. The composition of the new medium was then: 3 grams beef extract; 5 grams peptone, 5 grams lactose, 1 gram dextrose, 0.1 gram KH_2PO_4, 0.2 gram Na_2HPO .12 H_2O, 15 grams agar, and 1 liter distilled water.

A series of 79 milk samples, 51 raw and 28 pasteurized, were plated on three media to determine the efficiency of these media under actual working conditions. The media used were standard agar, Devereux's medium, and the new medium devised as a result of the first part of the experimental work. Duplicate plates were poured for

each incubation temperature, thirty and thirty-seven degrees Centigrade. The procedure used was that contained in the Standard Methods of Milk Analysis (Fifth Edition) (36). The milk samples were obtained through the courtesy of Mr. J. King of the Northampton Board of Health. Efforts were made to obtain samples which would vary from very bad to very good milk.

In Table VI are shown the counts obtained from 79 different samples of milk from each of the six combinations of incubation temperatures and media. The individual counts appearing in this table are the arithmetical averages of duplicate plates. The thirty-seven degrees Centigrade count on plain agar was taken as the standard and any variation above or below this count is indicated by a plus or a minus sign.

The buffering index of this new medium is 1.2 as compared to 0.9 for Devereux and 0.65 for the American Public Health Association standard medium, all measured at a pH of 5.0 to 8.0, according to Brown's method (13).

TABLE VI

RESULTS OF PLATING MILK SAMPLES

Sample.	Temperature	Plain agar.	Difference.	%	Dever-eux.	Difference.	%	Lactose	Difference.	%
RAW										
1	37	7000	0000	100	7000	0	100	9000	2000	129
	30	15000	80000	214	16000	9000	228	16000	9000	228
2	37	127000	0000	100	20000	107000	16	28000	99000	22
	30	18000	109000	14	18000	109000	14	23000	104000	18
3	37	16000	0000	100	20000	107000	138	24000	8000	150
	30	93000	77000	581	79000	53000	431	100000	84000	625
4	37	21000	0000	100	27000	6000	129	27000	6000	129
	30	22000	1000	105	35000	14000	167	40000	19000	190
5	37	530000	0000	100	860000	330000	162	720000	190000	136
	30	750000	220000	142	790000	260000	150	970000	440000	183
6	37	360000	0000	100	480000	120000	133	420000	60000	117
	30	720000	360000	200	380000	20000	105	440000	80000	122
7	37	22000	0000	100	16000	6000	73	20000	2000	91
	30	21000	1000	95	18000	4000	82	22000	0000	100
8	37	120000	0000	100	16000	51000	57	68000	52000	57
	30	114000	6000	95	81000	39500	68	76000	44000	63
9	37	36000	0000	100	20000	16000	55	30000	6000	83
	30	33000	3000	92	25000	11000	69	38000	2000	106
10	37	2000	0000	100	15000	5000	75	31000	11000	155
	30	23000	3000	115	16000	4000	80	27000	7000	135
11	37	56000	0000	100	60000	2000	103	65000	7000	112
	30	59000	1000	102	53000	5000	91	78000	20000	135
12	37	390000	0000	100	360000	30000	92	420000	30000	108
	30	420000	30000	108	320000	70000	82	460000	70000	118
13	37	16000	0000	100	13000	3000	81	15000	1000	94
	30	26000	10000	163	13000	3000	81	22000	6000	138
14	37	440000	0000	100	110000	330000	25	240000	200000	54
	30	350000	90000	79	130000	310000	29	190000	250000	43
15	37	17000	0000	100	12000	5000	71	22000	5000	129
	30	30000	13000	177	23000	6000	135	20000	13000	177
16	37	149000	0000	100	139000	10000	93	139000	10000	93
	30	196000	47000	132	160000	11000	107	177000	28000	119
17	37	30000	0000	100	28000	2000	93	32000	2000	107
	30	44000	14000	147	35000	5000	117	46000	16000	153

TABLE VI (continued)

Sample	Temperature	Plain agar	Difference.	%	Deveraux	Difference	%	Lactose	Difference.	%
RAW										
18	37	44000	0000	100	139000	10000	93	139000	10000	93
	30	56000	12000	127	52000	11000	107	177000	28000	119
19	37	7600	0000	100	8300	7000	109	11700	4100	154
	30	13300	5700	175	13900	6300	183	15000	7400	197
20	37	97000	0000	100	57000	40000	49	4000	57000	41
	30	246000	149000	254	170000	73000	175	275000	178000	284
21	37	270000	0000	100	310000	40000	115	280000	10000	104
	30	710000	440000	263	710000	440000	263	820000	550000	304
22	37	59000	0000	100	43000	4000	93	74000	15000	125
	30	125000	66000	212	83000	24000	141	118000	59000	200
23	37	142000	0000	100	156000	14000	110	137000	5000	96
	30	158000	16000	111	449000	7000	105	157000	15000	111
25	37	23000	0000	100	18000	5000	78	26000	3000	113
	30	33000	10000	144	24000	1000	104	34000	11000	148
26	37	17000	0000	100	17000	0000	100	20000	3000	118
	30	30000	13000	117	21000	4000	124	13000	4000	76
27	37	19000	0000	100	15000	4000	79	21000	2000	111
	30	34000	15000	179	21000	2000	111	30000	11000	158
28	37	12000	0000	100	12000	0000	100	13000	1000	108
	30	20000	8000	167	12000	0000	100	21000	9000	175
29	37	70000	0000	100	60000	10000	86	60000	10000	86
	30	110000	40000	157	80000	10000	114	110000	40000	157
30	37	5000	0000	100	3000	3000	60	4000	1000	80
	30	8000	3000	160	6000	1000	120	9000	4000	180
PASTEURIZED										
31	37	62000	0000	100	5000	57000	8	87000	15000	124
	30	66000	4000	106	6000	56000	10	87000	15000	124
32	37	20000	0000	100	17000	3000	85	94000	74000	470
	30	27000	7000	135	21000	1000	105	97000	77000	485
33	37	15000	0000	100	9000	6000	60	8000	7000	53
	30	103000	88000	686	58000	43000	386	78000	63000	520
34	37	1000	0000	100	800	200	80	1600	600	160
	30	6800	5800	680	4600	3600	460	6400	5400	640
35	37	2200	0000	100	1700	500	77	3400	1200	155
	30	5600	3400	254	2900	700	132	7000	4800	318

TABLE VI (continued)

Sample	Temperature.	Plain agar	Difference	%	Deveren.	Difference.	%	Lactose	Difference.	%
PASTEURIZED										
36	37	10300	0000	100	800	9500	8	1000	9300	10
	30	10800	500	105	3500	6800	34	14900	4600	145
37	37	88000	0000	100	144000	56000	164	335000	147000	267
	30	285000	197000	324	230000	142000	261	365000	277000	414
38	37	200000	0000	100	40000	160000	20	47000	153000	23
	30	52000	148000	26	34000	166000	17	73000	127000	36
39	37	54000	0000	100	8000	46000	15	6000	48000	11
	30	171000	117000	317	78000	24000	145	147000	93000	272
40	37	32500	0000	100	2000	36000	15	11000	27000	29
	30	24000	8500	74	3600	28900	11	15700	16800	48
41	37	38000	0000	100	2000	36000	5	11000	27000	29
	30	2000	3600	5	2000	36000	5	3000	35000	8
42	37	24000	0000	100	3000	21000	12	16000	8000	67
	30	12000	12000	50	1000	23000	4	15000	9000	63
43	37	12000	0000	100	2500	9500	21	2700	93000	23
	30	17100	5100	142	7000	5000	58	19600	7600	163
44	37	95000	0000	100	7000	88000	7	13000	82000	14
	30	31000	64000	32	26000	69000	23	35000	60000	37
45	37	30000	0000	100	10300	19700	68	16000	14000	53
	30	25500	4500	85	4300	25700	14	17800	12200	59
RAW										
46	37	12100	0000	100	4500	7600	37	5900	6200	49
	30	k4800	2700	122	10500	1600	87	13400	1300	111
47	37	25700	0000	100	7400	18300	29	4700	21000	18
	30	21000	4000	82	17800	7900	69	24100	1600	94
48	37	32000	0000	100	12900	19100	40	10800	21200	34
	30	25200	6800	79	21300	10700	67	26800	5200	84
49	37	11500	0000	100	7700	3800	67	8200	3300	78
	30	15600	4100	136	13400	1900	117	16900	5400	147
50	37	28000	0000	100	9000	21000	32	9000	19000	32
	30	18000	10000	64	25000	3000	89	37000	9000	132
52	37	64000	0000	100	41000	23000	64	164000	100000	256
	30	134000	70000	209	162000	98000	253	198000	134000	309
53	37	5000	0000	100	9000	4000	180	6000	1000	120
	30	19000	14000	380	15000	10000	300	23000	18000	460

TABLE VII (continued)

Sample	Temperature	Plain agar	Difference	%	Dever-eux	Difference.	%	Lactose	Difference.	%
RAW										
54	37	6000	0000	100	3000	3000	50	2000	4000	33
	30	4000	2000	66	3000	3000	50	4000	2000	66
55	37	14000	0000	100	9000	5000	64	12000	2000	86
	30	22000	8000	157	13000	1000	93	28000	14000	200
56	37	6000	0000	100	6000	0000	100	9000	3000	150
	30	12000	6000	200	13000	7000	217	16000	10000	267
57	37	11000	0000	100	52000	41000	472	37000	26000	337
	30	48000	37000	436	62000	51000	563	69000	58000	627
59	37	48000	0000	100	20000	28000	42	14000	34000	29
	30	104000	56000	217	47000	1000	98	124000	76000	258
60	37	33000	0000	100	17000	16000	51	12000	21000	36
	30	113000	80000	342	45000	12000	136	106000	73000	321
61	37	61000	0000	100	17000	44000	28	24000	37000	39
	30	133000	72000	218	91000	30000	149	123000	62000	202
62	37	13000	0000	100	8000	5000	62	7000	6000	54
	30	49000	36000	477	31000	18000	238	53000	40000	408
63	37	45000	0000	100	44000	1000	98	42000	3000	93
	30	55000	10000	122	58000	13000	129	49000	4000	109
64	37	185000	0000	100	69000	116000	37	183000	2000	99
	30	159000	26000	86	139000	46000	75	214000	29000	116
65	37	39000	0000	100	34000	5000	87	34000	5000	87
	30	191000	152000	490	169000	130000	432	205000	166000	526
PASTEURIZED										
66	37	5000	0000	100	3100	1900	62	2000	3000	60
	30	17100	12100	342	12100	7100	232	16600	11600	332
RAW										
67	37	50000	0000	100	15000	35000	30	17000	33000	34
	30	103000	53000	206	36000	14000	72	105000	55000	210
68	37	6000	0000	100	6000	0000	100	4000	2000	27
	30	17000	11000	283	14000	8000	234	14000	8000	234
69	37	24000	0000	100	8000	16000	34	7000	17000	29
	30	62000	38000	259	39000	15000	163	81000	54000	325
70	37	21000	0000	100	8000	13000	38	7000	14000	33
	39	56000	35000	267	21000	0000	100	56000	35000	267

TABLE VI (Continued)

Sample	Temperature.	Plain agar	Difference.	%	Dever-eux.	Difference.	%	Lactose	Difference	%
PASTEURIZED										
71	37	2000	0000	100	1000	1000	50	1000	1000	50
	30	19000	17000	950	5000	3000	250	16000	14000	800
72	37	7000	0000	100	2000	5000	29	5000	2000	71
	30	12000	5000	171	6000	1000	86	15000	8000	214
73	37	6000	0000	100	3000	3000	50	5000	1000	16
	30	11000	5000	183	2000	4000	67	17000	11000	184
74	37	6000	0000	100	3000	3000	50	3000	3000	50
	30	6000	0000	100	6000	0000	100	17000	11000	184
75	37	4000	0000	100	2000	2000	50	4000	0000	100
	30	9000	5000	225	6000	2000	150	17000	13000	426
76	37	1800	0000	100	11200	9400	622	0000	0000	000
	30	14300	12500	795	26200	24400	1458	40000	38200	2220
77	37	1800	0000	100	19700	17900	1095	0000	0000	000
	30	10400	8600	577	31500	29700	1750	36000	34200	2000
78	37	2500	0000	100	10300	7800	412	25000	22500	1000
	30	10100	7600	404	21100	19600	845	23500	20500	1000
79	37	2400	0000	100	14300	11900	596	38000	35600	1582
	30	10400	8000	434	10400	8000	434	17700	15300	738
80	37	2700	0000	100	8300	5600	307	30000	27300	1110
	30	9600	6900	356	13200	10500	490	14700	12000	545
81	37	5400	0000	100	18200	12800	337	0000	0000	000
	30	10300	4900	191	18900	13500	350	30000	24600	556
82	37	1100	0000	100	7900	6800	718	40000	38900	3640
	30	2200	1100	200	4600	3500	418	10800	9700	980

TABLE VII

Summary of Counts.

Medium and Incubation Temperature (Centigrade)	Counts Expressed as Percent *	Total number of highest Counts (all cases considered)	Total number of lowest Counts (all cases considered)
Raw			
37° (Standard	100	7	9
37° (Devereux	84	0	22
37° (Modified lactose	94	1	16
30° (Standard	186	13	1
30° (Devereux	146	1	2
30° (Modified lactose	200	29	1
Total		51	51
Pasteurized			
37° (Standard	100	6	8
37° (Devereux	180	0	11
37° (Modified lactose	366	4	5
30° (Standard	284	5	0
30° (Devereux	297	0	4
30° (Modified lactose	480	13	0
Total		28	28
Combined **			
37° (Standard	100	13	17
37° (Devereux	118	0	33
37° (Modified lactose	183	5	21
30° (Standard	221	18	1
30° (Devereux	199	1	6
30° (Modified lactose	299	42	1
Total		79	79

* Standard agar at 37° C. is taken as 100%; all results on other media are expressed in terms of percentage of counts on standard agar at 37° C.

**All samples, both raw and pasteurized.

Analysis of Table VII

There are several ways of making a statistical analysis of the foregoing data but the following was considered best: The standard agar count at thirty-seven degrees Centigrade was taken as 100 per cent. The bacteria counts on all other media and at the other temperatures were then calculated for the same sample of milk as percentages of the count on the standard medium. The counts of one sample bear no relation to the counts of another sample of milk; to average these counts, therefore, would be illogical and of no value. The percentage variations of the different counts, however, should be a function of the media and temperature of incubation. In the long run, a medium and an incubation temperature should produce rather constant increases or decreases for all milk samples. The counts, therefore, were expressed as percentages of count on the standard medium at thirty-seven degrees Centigrade, and averaged. The outstanding features shown by Table VII are: from all samples higher counts were obtained on a medium, and at a temperature other than the thirty-seven degrees Centigrade count on standard agar, excepting from raw milk and on the new agar and on Devereux's agar at thirty-seven degrees Centigrade. Comparing the effects of incubation temperatures with all media, it was found that from raw milk 84 per cent of the highest counts were obtained at thirty degrees Centigrade and 16 per cent at thirty-seven degrees Centigrade. From pasteurized milk 64 per cent of the highest counts were obtained at thirty degrees Centigrade and 36 per cent at thirty-seven degrees Centigrade. From all samples consid-

ered together, 10 per cent of the lowest counts were obtained at thirty degrees Centigrade and 90 per cent at thirty-seven degrees Centigrade.

The results on the various media compared at all temperatures showed that: 39 per cent of the highest counts were on standard agar, 1 per cent of the highest counts were on Devereux's agar, 60 per cent of the highest counts were on the new agar; 23 per cent of the lowest counts were obtained on standard agar, 49 per cent of the lowest counts were obtained on Devereux's agar, and 28 per cent of the lowest counts were obtained on the new agar.

From these data it appears that the new medium at thirty degrees Centigrade has distinct advantages over the other combinations of media and incubation temperature studied. Devereux's medium has few advantages over the standard medium, and none over the new medium. The counts at thirty-seven degrees Centigrade on all media are decidedly unfavorable in comparison with the counts at thirty degrees Centigrade.

It therefore seems very desirable that an incubation temperature of thirty degrees Centigrade for forty-eight hours be used,and a fermentable carbohydrate, preferably lactose, be added to a medium containing enough buffering capacity to take care of the acid products of bacterial metabolism.

DISCUSSION

The criticisms directed against the standard medium are based on the contention that it does not support the growth of all types of organisms found in milk, and also that the temperature of incubation is not optimum for the organisms commonly found in milk.

The original purpose of this investigation was to compare the merits of the standard agar medium of the American Public Health Association, and the Devereux yeast extract medium for plating milk samples. It was immediately evident there was a need for a more thorough investigation of the nutritive requirements of the bacteria commonly found in milk. It was hoped that from this study a new medium could be devised, and a more suitable incubation temperature arrived at than those at present in use for plating milk samples.

The constituents of Devereux's medium were added separately and in combination to the standard medium in order to determine if any of the ingredients could affect growth of certain of the common milk organisms. Since lactose had been recommended by many investigators, it was decided to incorporate it as one of the ingredients to be tested. The organisms used consisted of members of the subtilis group, coli-aerogenes group, and a group of miscellaneous cocci commonly found in milk. These organisms were plated out from a dilution that would not result in overcrowding, and were incubated at thirty and at thirty seven degrees Centigrade for forty-eight hours. The basis for comparison was colony growth as measured by size of colony. Standard agar was used as a control. Production of acid as indicated by the color changes in brom thymol blue was

observed, as preliminary work had indicated that the production of acid might, in some cases, be correlated with growth.

The results will be discussed for each group of organisms. In the subtilis group it was found that, in general, yeast extract exerted no stimulative action on growth, dextrose exerted an inhibitive effect due to the production of acid, Devereux's medium inhibited growth, the substitution of lactose for dextrose in Devereux's medium was of little or no value, and lactose exerted no inhibitive action. In this group the production of acid is a determining factor in growth.

In the coli-aerogenes group, the addition of dextrose decreased the size of the colonies slightly, yeast extract produced no change, the combination of yeast extract and dextrose produced no change in growth, Devereux's medium increased growth slightly, while lactose produced the largest increase in colony size of all the ingredients tested. The addition of a buffer did not increase growth on standard agar plus dextrose, Devereux's medium, or on modified Devereux's medium. This is thought to be due to the fact that the coli-aerogenes group produces such large quantities of acid that a much larger quantity of buffer salts was needed than the amount furnished, in order to prevent a lowering of the H-ion concentration.

In the group of miscellaneous cocci; the standard medium was the least favorable medium for growth in this group. The addition of dextrose and yeast extract, singly and in combination, caused an increase in growth in most cases. The addition of lactose increased growth in all

cases and, significantly, was the only ingredient that produced an increase in growth of the Streptococcus lactis. The addition of a buffer salt did not stimulate growth to any extent.

Summing up the effect of the ingredients on certain bacteria commonly found in milk it is evident that: dextrose stimulated growth in a few cases, and inhibited growth in a large majority of the cases; yeast extract stimulated growth in a few cases, and had little, or no, effect in the majority of cases; Devereux's medium stimulated growth in a few cases, and inhibited growth in a majority of the cases; lactose stimulated growth in the majority ofthe cases, and inhibited in none; thirty degrees Centigrade was a more desirable tempera- of incubation than thirty seven degrees Centigrade, except for the coli-aerogenes group; with this group thirty-seven degrees Centigrade appeared to be slightly the better.

The failure of dextrose to stimulate growth is thought to be due to the production of acid. Berman and Rettger (3) have proved that increased H-ion concentration inhibits nitrogen metabolism. The use of buffer salts prevents irregularities in counts due to the domination of any one group of bacteria, which, by the production of acid, may inhibit the other organisms not aciduric. Mallman and Gallo (23) have shown that the addition of a buffer salt increases bacterial growth.

A new medium was made up for the second part of the investigation. The constituents of this medium were: 3 grams of beef extract, 5 grams of peptone, 5 grams of lactose, 1 gram of dextrose, 0.1 gram of KH_2PO_4, and 0.2 gram of $Na_2HPO_4.12H_2O$. This new medium was compared with

Devereux's and the standard American Public Health Association agar media, using bacterial counts on milk samples as the basis of comparison. Plates were incubated at thirty seven and at thirty degrees Centigrade for forty-eight hours. Thirty degrees Centigrade was chosen as a temperature of incubation because the work of the first part of the investigation had revealed that thirty degrees Centigrade was as good as, or better than, thirty seven degrees Centigrade. Fifty-one samples of raw, and twenty-eight of pasteurized milk were plated.

This part of the investigation indicated that an incubation temperature of thirty degrees Centigrade is much more desirable than thirty seven degrees Centigrade. Referring to Table VII, all samples combined, it will be noticed that all of the counts at thirty degrees Centigrade were much higher than any of the counts at thirty seven degrees Centigrade. Devereux's medium was not better than the standard medium, inasmuch as the counts on this medium were not consistently higher than the counts on the standard medium. The new medium proved to be an improvement over the other media studied, since the counts were, for the most part, consistently higher than those on any other medium. Referring to the table on combined counts on raw and pasteurized milk, the new medium gave the highest counts at either temperature. The new medium at thirty degrees Centigrade gave the highest percentage count. The value of this new medium lies not only in the kind and amount of carbohydrate, but in the buffering quality of the medium. This latter factor did much to reduce irregularities in counts. It is thought that this medium and an incubation

temperature of thirty degrees Centigrade will give more accurate and comparative counts, since it gives higher counts and does not appear to inhibit bacterial growth.

We believe that the standard method for plating technic can be relieved of some of its inaccuracies by the substitution of the new medium, described here, for the standard agar now in use. A further improvement of the technic can be obtained by the use of thirty degrees Centigrade instead of thirty seven degrees Centigrade for incubation. The increased size of the colonies resulting from the use of the new medium and an incubation temperature of thirty degrees Centigrade should increase the ease of counting the plates.

SUMMARY

From the data obtained in this investigation the following summary appears to be justified:

(1) Yeast extract did not stimulate growth of the majority of the organisms employed in this study.

(2) The addition of lactose and dextrose stimulated growth of all but a few of the organisms employed. Lactose exerted a greater stimulating effect than dextrose.

(3) The growth of certain of the organisms was inhibited by their production of acid in the media during incubation; the inhibition was probably due to the lowering of nitrogen metabolism because of the production of acid.

(4) The addition of a buffer prevented acid production and the consequent inhibition of growth.

(5) A new medium was devised containing both dextrose and lactose, and a buffer.

(6) The new medium was compared with the standard American Public Health Association and Devereux media, and produced larger colonies than either, thus increasing the ease and accuracy of counting the plates.

(7) Thirty degrees Centigrade was found to be a more favorable incubation temperature than thirty-seven degrees Centigrade.

(8) Devereux's reported results which he obtained by comparing bacterial counts of milk on the standard agar and his yeast extract medium were not confirmed.

(9) The higher counts and larger colonies were obtained when the new medium was used for plating milk samples indicated that this medium may be recommended as a substitute for media now employed.

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We, the undersigned, members of Mr. Supowitz's thesis committee, approve this thesis.

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